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(54) **Title:** CARBODIIMIDE CROSS–LINKED ALBUMIN FOR BIOADHESIVES, SURGICAL SEALANTS AND IMPLANTABLE DEVICES

(57) **Abstract**

The invention provides methods and products relating to protein–based bioadhesives and surgical sealants, and implantable devices for drug delivery and prostheses. In particular, the invention relates to albumin–based methods and products. Methods and products of the invention are useful for adhering biological tissues to each other or to prosthetic devices, for sealing fluid or gaseous leaks in biological tissues, or for preparing bio–erodable devices. Methods and products of the invention are particularly useful in cardiovascular and pulmonary applications.

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CARBODIIMIDE CROSS-LINKED ALBUMIN FOR BIOADHESIVES, SURGICAL SEALANTS AND IMPLANTABLE DEVICES

This application claims the benefit of USSN 60/090,609, filed on June 23, 1998, the disclosure of which is incorporated herein by reference.

Field of the Invention

The present invention is directed to the development and use of carbodiimide cross-linked proteins for use *in vitro* or *in vivo* during experimental or surgical procedures to bond a tissue to another tissue and/or to a prosthetic device; to seal incisions, perforations, and/or fluid or gaseous leaks in tissues; or to form implantable devices for drug delivery or prostheses, or to form coatings on inert devices for biocompatibility and other functions.

Background of the Invention

Biomedical researchers have attempted to develop various methods and products to bind tissues to tissues, and/or tissues to prosthetic devices, for a wide array of *in vitro* or *in vivo* experimental or surgical procedures, including the implantation of devices such as prostheses for drug delivery systems. In particular, researchers have attempted to develop safe and efficient means to bind or seal tissues cut, torn or perforated as a result of trauma and/or surgical procedures.

Historically, suturing and stapling have been the most common methods for binding or sealing tissues. There are, however, a number of disadvantages to these methods. For example, depending upon the nature of the tissues and the extent of tissue damage, suturing and stapling may require significant skill and/or extensive surgical time. More important, the results are often less than satisfactory due to gaps between sutures or staples, tearing of delicate tissue around sutures or staples, and the possibility of progressive weakening of the bindings that may result in leaks of biological fluids or bacterial infections.

For these and other reasons, safer and more efficient methods have been sought to bind or seal tissues. In particular, bioadhesives or surgical sealants have been developed to adhere to tissue surfaces and to form bonds between tissues until healing is complete.

For example, fibrin-based adhesives have commanded considerable attention in this area (see, e.g., Epstein et al. (1986), *Ann. Otol. Rhinol. Laryngol.* 95:40-45; Siedentop et al. (1983), *Laryngoscope* 93:1310-1313). Fibrin adhesives, however, have low mechanical (bond) strength, as well as a lengthy set-up time, which limits their utility.

A variety of approaches have been developed to utilize collagen, the major connective tissue protein in animals, as a bioadhesive or surgical sealant. However, success has generally been limited by various deficiencies, including cross-linking/polymerization reactions that are exothermic, long reaction times, and reactions that are inoperative in the presence of oxygen or at physiological pH ranges (see, e.g., Lee et al. (1970), in *Adhesion in Biological Systems,* R. S. Manly, ed., Academic Press, New York, Chap. 17). Moreover, some prior art collagen-based adhesives have contained toxic materials or by-products, rendering them inappropriate for *in vivo* biomedical use (see, e.g., Buonocore (1970), in *Adhesion in Biological Systems*, R.S. Manly, ed., Academic Press, New York, Chap. 15, and U.S. Pat. No. 3,453,222). More recent approaches employing collagen-based bioadhesives may be found in, e.g., U.S. Pat. No. 5,219,895, U.S. Pat. No. 5,354,336, U.S. Pat. No. 5,874,537, and PCT Publication WO 97/42986.

Non-biological adhesives such as cyanoacrylates (e.g., isobutyl-2-cyanoacrylate) have also been examined for their potential adhesive properties. However, these materials are difficult to apply *in vivo* because cyanoacrylate adhesives require application in a dry field. Furthermore, the cured solid form of cyanoacrylate produced in this approach is non-resorbable, therefore limiting its usefulness for *in vivo* applications. In addition, as a result of the exothermic nature of the polymerization reaction, adverse tissue effects have been reported.

A bioadhesive based upon gelatin-resorcinol cross-linked with formaldehyde has also been developed (see, e.g., Koehnlein et al. (1969), *Surgery* 66:377-382, and Bellotto et al. (1992), *Surg. Gynecol. Obstet.* 174:221-224), but has similar disadvantages. Of particular concern with such products is the hazardous nature of the formaldehyde and resorcinol. Furthermore, the gelatin must be applied at a temperature significantly above the human body

temperature, and requires significant attention during mixing and application to achieve successful results.

Carbodiimides have been utilized in a variety of contexts as cross-linking reagents. For example, carbodiimides have been used as cross-linking reagents with the polysaccharides hyaluronic acid and pectin (see, e.g., Tomihata et al. (1997), *J. Biomed. Mater. Res.* 37:243-251). In addition, carbodiimides were used to cross-link aqueous mixtures of gelatin and poly(L-glutamic acid) (PLGA) as adhesive agents (see, e.g., Otani et al. (1996), *J. Biomed. Mater. Res.* 31:157-166). However, one of the more disadvantageous results was the potentially cytotoxic effect of the derivatives and remaining products of the reaction.

At present, few adhesive compositions exist that have utility in surgical and biomedical applications, particularly those involving soft tissues. There remains a need, therefore, for safe, effective compositions for use as bioadhesives, surgical sealants, and/or implantable devices.

Summary of the Invention

The invention provides methods and compositions that are useful for adhering biological tissues and/or prosthetic devices, sealing fluid and/or gaseous leaks in biological tissues, and preparing implants for drug delivery, including bio-erodable implants.

In one aspect, the invention comprises protein and cross-linking preparations that are useful for cross-linking biological tissues and/or prosthetic devices. Compositions of the invention comprise protein solutions that are suitable for tissue cross-linking.

In preferred embodiments, protein preparations of the invention comprise albumin preparations. In one embodiment the albumin is bovine serum albumin (BSA). In a preferred embodiment, the albumin is a human albumin. Preferably, the albumin preparations are provided at a pH of between 5.0 and 8.0, more preferably between 5.5 and 7.5.

In one embodiment, the albumin preparation comprises a protein that is a naturally occurring albumin protein, a recombinant albumin protein, a major fragment of an albumin protein, or a chemically modified albumin. In a preferred embodiment, the albumin is a mammalian albumin protein or a major fragment of a mammalian albumin protein. Most preferably, the albumin protein comprises an amino sequence of at least 100 amino acid residues having at least 60% homology to an amino acid sequence of human albumin.

In alternative embodiments, the albumin comprises an amino-acid sequence which has been recombinantly modified relative to a naturally occurring albumin sequence to enhance one or more physical properties such as solubility, reactivity with a carbodiimide cross-linker, stability, viscosity in an aqueous solution, and immunocompatibility. In further embodiments, the albumin preparation comprises additives to increase or decrease the crosslinking reaction rate or to promote interaction between the protein solution and the tissue or material at the site of application. For example, the albumin may provided along with one or more surfactant, lipid, and/or fatty acid.

In any one of the above embodiments, the albumin may be covalently bound to a molecule selected from the group consisting of polysaccharides (e.g., glycosaminoglycans, dextrans, hyaluronic acid, chondroitin sulfate, heparan sulfates), polyethers (e.g., polyethylene glycol, polypropylene glycol, polybutylene glycol), polyesters (e.g., polylactic acid, polyglycolic acid, polysalicylic acid), and aliphatic, alicyclic, aromatic, perfluorinated or non-perfluorinated, and acylating or sulfonating agents. In addition the albumin preparation may contain a chlorinated, fluorinated, brominated or iodinated albumin protein.

Preferably, the albumin protein is provided in an aqueous solution at a concentration of about 10-50 % by weight, more preferably about 20-40% by weight, most preferably 35-40% by weight. In an alternative embodiment, the albumin protein is provided in solution of secondary or a tertiary alcohol. In a preferred embodiment, the alcohol solution comprises isopropyl alcohol (IPA) or isobutyl alcohol (IBA). In a most preferred embodiment, the albumin is dissolved in a 20% IPA solution or an 8% IBA solution.

In further embodiments, the protein may be derivatized to alter its properties in the cross-linking reaction or to promote its interaction with tissue or substrate at the site of application, as explained and illustrated in the following description and examples.

In preferred embodiments, the cross-linker preparation comprises a carbodiimide having a general structure R_1-N=C=N-R_2 wherein R_1 and R_2 are independently selected from the group consisting of straight chain or branched, saturated or unsaturated, alkyl, alkenyl, aryl, aralkyl, or aralkenyl groups, or variants thereof with halogen, tertiary amino, ester, keto substituents or other degradable groups. More preferably, the carbodiimide is selected from the group consisting of ethyl dimethylaminopropyl carbodiimide (EDC·HCl); 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; 1,3-di-*p*-tolylcarbodiimide; 1,3-

diisopropylcarbodiimide; 1,3-dicyclohexylcarbodiimide; 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate; polycarbodiimide; 1-tert-butyl-3-ethylcarbodiimide; 1,3-dicyclohexylcarbodiimide; 1,3-bis(trimethylsilyl)carbodiimide; 1,3-di-tert-butylcarbodiimide; 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide; and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. Most preferably, the carbodiimide is EDC·HCl. In preferred embodiments, the carbodiimide preparation comprises between 10% and 20% EDC·HCl, most preferably 15% EDC·HCl.

In another aspect, the invention provides a method for producing a cross-linked albumin composition for use in a bioadhesive, surgical sealant or implantable device, comprising the steps of providing an albumin preparation; providing a carbodiimide preparation; and mixing the albumin preparation and the carbodiimide preparation under conditions which permit cross-linking of the albumin. In preferred embodiments, the albumin and carbodiimide preparations are mixed in one of the following ratios: between approximately 1:1 and 1:100, between approximately 1:5 and 1:50, between approximately 1:10 and 1:20, between approximately 100:1 and 1:1, between approximately 36:1 and 4:1, and approximately 18:1.

Preferred methods of the invention are useful for sealing incisions, perforations, and/or fluid or gaseous leaks in biological tissues during a surgical procedure, and comprise contacting the tissue with an effective amount of an albumin preparation and a carbodiimide preparation under conditions that promote cross-linking of the albumin preparation to the tissue thereby sealing the incision, perforation, or fluid or gaseous leak. Such methods are particularly useful for surgical procedures such as cardiovascular, pulmonary, renal, and hepatic surgeries.

According to methods of the invention, a fluid or gaseous leak can be sealed by cross-linking the tissues surrounding the leak. Alternatively, a cross-linked gel of the invention can seal a leak by physically occluding it without cross-linking the surrounding tissues.

In one embodiment, the albumin and carbodiimide preparations are mixed before applying them to a tissue locus. Alternatively, the mixing step is performed at a tissue locus. In an alternative embodiment, an accessory molecule is provided and mixed with the albumin and carbodiimide preparations. The accessory molecule is preferably selected from the group consisting of viscosity-enhancing agents, cross-linkers, buffers, hormones, growth factors, antibiotics, surfactants, lipids, fatty acids, and anti-inflammatory agents.

In a preferred embodiment, a primer solution is applied to the tissue or prosthetic device before adding the mixture of albumin and cross-linker. The primer solution may be a saline solution. Preferably, the primer solution is an albumin solution. More preferably, the primer solution is identical to the albumin preparation used in the cross-linking reaction, and most preferably the primer solution is a dilute solution of the albumin preparation.

In any on of the above embodiments, the albumin and carbodiimide preparations are preferably provided at a pH between 5.0 and 8.0, more preferably between 5.5 and 7.5, most preferably between 6.0 and 7.0. However, the pH of the preparations is preferably adapted to the desired rate of cross-linking. Rapid cross-linking is useful in cardiovascular applications to rapidly seal blood leaks. Rapid cross-linking involvin EDC·HCl is obtained at pH 5.0 to 5.5. Alternatively, for pulmonary applications, rapid cross-linking is not as important and the preparations can be provided at a higher pH. The cross-linking reaction rates can also be modified using additives, derivatized albumin, or by altering the ratio or concentrations of albumin and carbodiimide, as described in the following description and as illustrated by the following examples.

In further embodiments, the preparations may be provided in hydrophobic solutions. Preferably, the solutions do not interfere with the cross-linking reaction. Preferred solutions include secondary and tertiary alcohols, including IPA and IBA. In preferred applications, a 30% solution of BSA is made using 20% IPA or 8% IBA.

In other embodiments of the invention, the protein and cross-linker may be provided as dry powders. The protein and cross-linker powders are preferably mixed prior to applying them to a tissue site. In a most preferred embodiment, the protein and cross-linker absorb body fluids from the site of application, and this fluid absorption starts the cross-linking reaction.

Alternatively, additional fluid may be provided as necessary to the site of application. In a further embodiment, the site of application is primed with a solution of saline or dilute protein before the dry protein and cross-linker mixture is applied. The primer solution is then absorbed by the dry mixture, thereby starting the cross-linking reaction.

In another aspect, the invention provides methods and compositions that bind or adhere to synthetic material such as artificial blood vessels (for example PTFE material) or biological implants (for example polyethylene material).

In a related aspect, the invention provides a method for adhering a first biological tissue to a second tissue and/or prosthetic device, comprising contacting the first tissue and the second tissue or prosthetic device with a mixture of an albumin preparation and a carbodiimide preparation under conditions that promote cross-linking of said albumin preparation to the first tissue and second tissue or prosthetic device.

In a further aspect of the invention, methods are provided for forming an implantable device comprising. The methods comprise providing an albumin preparation, providing a carbodiimide preparation, providing a mold, and mixing the albumin preparation and the carbodiimide preparation under conditions which permit cross-linking of the albumin in the mold.

Products of the invention also include a bioadhesive, surgical sealant or implantable device produced according to any of the foregoing methods.

In another aspect, the invention provides a kit for producing a bioadhesive, surgical sealant or implantable device comprising, in separate containers, an albumin preparation, and a carbodiimide preparation. In a preferred embodiment, the kit further comprises an accessory molecule, preferably a molecule selected from the group consisting of viscosity-enhancing agents, cross-linkers, buffers, hormones, growth factors, antibiotics, anti-inflammatory agents, hydrophobicity increasing agents, and surfactants.

In another embodiment of the kit, the albumin preparation and the carbodiimide preparation are provided in a binary delivery device that delivers the preparations in a ratio which is either predetermined or regulatable.

A detailed description of certain preferred embodiments of the invention is provided below. Other embodiments of the invention are apparent upon review of the detailed description that follows.

Detailed Description of the Invention

In general, the invention provides methods and products relating to protein-based bioadhesives and surgical sealants, and implantable devices for drug delivery and prostheses. In preferred embodiments, the invention relates to albumin-based methods and products. However, it should be understood that other proteins can be substituted in the following description of albumin-based methods and products. Compositions of the invention are formed by cross-

linking albumins by reacting them with carbodiimides under specified conditions. The general reaction proceeds in two steps (see, e.g., Damink et al., 1996). First, a free carboxyl group of an albumin molecule (e.g., the carboxy terminus, or a side chain of a glutamic or aspartic acid residue) attacks the carbodiimide to form one of two intermediates as follows:

$$X\text{-}COOH + R_1\text{-}N{=}C{=}N\text{-}R_2$$

$$\downarrow$$

$$X\text{-}(C{=}O)\text{-}O\text{-}(C{=}N\text{-}R_1)\text{-}NH\text{-}R_2 \; + \; X\text{-}(C{=}O)\text{-}O\text{-}(C{=}N\text{-}R_2)\text{-}NH\text{-}R_1$$

Next, a free amine group of the same or another albumin molecule (e.g., the amino terminus, or a side chain of a lysine or arginine residue) attacks one of these intermediates to yield the cross linked albumin and a substituted urea by-product as follows:

$$Y\text{-}NH_2 \; + \; X\text{-}(C{=}O)\text{-}O\text{-}(C{=}N\text{-}R_1)\text{-}NH\text{-}R_2$$

or

$$Y\text{-}NH_2 \; + \; X\text{-}(C{=}O)\text{-}O\text{-}(C{=}N\text{-}R_2)\text{-}NH\text{-}R_1$$

$$\downarrow$$

$$X\text{-}(C{=}O)\text{-}NH\text{-}Y \; + \; R_1\text{-}NH\text{-}(C{=}O)\text{-}NH\text{-}R_2$$

The reaction may be performed *in vitro* in a laboratory or manufacturing facility to produce products including cross-linked albumin as a bioadhesive or surgical sealant material, or as part of an implantable drug delivery device or prosthesis, or it may be performed *in vivo* during an experimental or surgical procedure to bond a tissue to another tissue and/or to a prosthetic device, or to seal incisions, perforations, and/or fluid or gaseous leaks in tissues. Optionally, and as described more fully below, the albumin starting material may be a chemically modified form of albumin, the carbodiimide cross-linker may include other functional or reactive groups, including additional carbodiimide groups such that the cross-linker is a poly(carbodiimide), additional non-carbodiimide cross-linkers may be employed, and/or various accessory molecules may be added to modify the course of the cross-linking reaction or the characteristics of the final product.

The methods of the invention comprise the steps of providing an appropriate albumin preparation and an appropriate carbodiimide, and optionally accessory molecules, and mixing these components under conditions which permit the carbodiimide to promote the formation of intermolecular (as well as intramolecular) cross-links amongst the albumin molecules. Appropriate albumin preparations, carbodiimide cross-linkers, reaction conditions, accessory molecules, and utilities are described in detail separately below. First, however, in order to more

clearly and concisely describe the subject matter which applicants regard as the invention, the following definitions are provided for specific terms used in the following written description and appended claims.

As used herein, the term "albumin" refers to any mammalian albumin protein, including allelic variants, and to modified albumins and albumin fragments which comprise a sequence of at least 100 amino acid residues having at least 60% homology, preferably at least 70 or 80% homology, to the human albumin sequence as disclosed in Minghetti et al. (1986) J. Biol. Chem. 261:6747-6757.

As used herein, the term "homology" means "homology" or "similarity" as calculated by the BLAST amino acid sequence comparison programs (including XBLAST, Altschul et al. (1990), J. Mol. Biol. 215:403-10) using default parameters for the appropriate programs (see http://www.ncbi.nlm.nih.gov). Alternatively, homology is calculated using the algorithm of Myers and Miller, CABIOS (1989), which is incorporated into the ALIGN program (version 2.0). Exemplary parameters for use when comparing amino acid sequences are a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4.

I. Albumin Preparations

The bioadhesives, surgical sealants, and implantable devices of the present invention comprise a cross-linked form of the protein albumin. Preferably, the albumin is of mammalian origin, but other sources of albumin also may be employed. It is believed that most albumins are readily cross-linked according to the methods of the invention. However, an albumin with low immunogenicity is preferred for *in vivo* applications. Accordingly, for uses in humans, it is preferred that the albumin is human albumin. Bovine serum albumin (BSA) may also be used in humans, and is more readily available. Alternatively, the albumin may be recombinant albumin, isolated from cells expressing a recombinant albumin gene, using methods known in the art. When produced recombinantly for use in humans, the albumin gene is preferably a human or bovine gene. However, other species or biosynthetic variants may be used. Major fragments of albumin, comprising at least 100 residues of an albumin sequence, whether produced by partial proteolysis or be recombinant means, may also be used instead of intact albumin. Alternatively, useful fragments may contain at least 50 residues, and more preferably at least 75 residues of an

albumin sequence. Finally, mixtures of different forms of albumin (e.g., human, bovine, recombinant, fragmented) may also be employed.

Albumin may be purified directly from tissues or cells, using methods well known in the art (see, e.g., Cohn et al. (1946) J. Amer. Chem. Soc. 68:459; Cohn et al. (1947) J. Amer. Chem. Soc. 69:1753; Chen (1967) J. Biol. Chem. 242:173). Alternatively, albumin may be purchased from a commercial supplier. For example, albumin preparations from various mammalian and avian species may be purchased from Sigma Chemical Company (St. Louis, MO) in the form of solutions or lyophilized powders. A preferred commercially available albumin preparation is a 30% human albumin solution (e.g., Sigma catalog no. A 9080) or a 30% BSA solution (e.g., Sigma catalog number A 8327).

In preferred embodiments, albumin is provided as an aqueous solution of 10-50%, preferably 20-40%, and most preferably about 35%-40% albumin by weight. As explained more fully below, lower concentrations of albumin may be employed when viscosity-enhancing agents are added. Preferably, the solution is substantially purified to remove contaminants such as immunogens or proteases which would disrupt or interfere with the bioadhesive or sealant properties of the cross-linked albumin. On the other hand, the presence of many other proteins, such as collagen, elastin, laminin, fibrin, and thrombin, can be tolerated.

Alternatively, albumin may be provided as a dry powder. In suchembodiments, the dry albumin is solubilized at the site of administration. Thus, body fluids (such as blood) present at the site of administration maybe sufficient to solubilize the protein. Alternatively, additional fluids may be provided along with the dry albumin. The cross-linker may also be provided as a dry powder that is solubilized at the site of administration. In a preferred embodiment, the dry protein and cross-linker are mixed prior to administration. In a most preferred embodiment, a wetting reagent is added to the protein and cross-linker mixture in order to increase fluid absorbance. Preferably, the wetting reagent absorbs water from the available body fluids and speeds up solubilization of the protein and cross-linker.

Albumin may be modified or derivatized to increase viscosity. For example albumin viscosity may be increased by covalently attaching relatively large (10-100 kD), substantially linear molecules such as polysaccharides (e.g., glycosaminoglycans, dextrans, hyaluronic acid, chondroitin sulfate, heparan sulfates), polyethers (e.g., polyethylene glycol, polypropylene glycol, polybutylene glycol), polyesters (e.g., polylactic acid, polyglycolic acid, polysalicylic

acid), and aliphatic, alicyclic or aromatic acylating or sulfonating agents. Preferred acylating agents including aliphatic, alicyclic and aromatic anhydrides or acid halides, particularly acid anhydrides of dicarboxylic acids. Non-limiting examples of these include glutaric anhydride, succinic anhydride, lauric anhydride, diglycolic anhydride, methacrylic anhydride, phthalic anhydride, succinyl chloride, glutaryl chloride, and lauroyl chloride. The acylating agents may also include various substituents and secondary functionalities such as aliphatic, alicyclic, aromatic and halogen substituents, as well as amino, carboxy, keto, ester, epoxy, and cyano functionalities, and combinations thereof. Similarly, preferred sulfonating agents useful in the invention include aliphatic, alicyclic and aromatic sulfonic acids and sulfonyl halides, which may also include various substituents and secondary functionalities as described above.

Albumin also may be modified or derivatized to increase its hydrophobicity in order to promote interactions with hydrophobic tissues or prosthetic materials. Specifically, albumin may be derivatized with branches or straight chain alkyl, alkenyl, or aromatic reagents, including long chain alkyl or alkenyl and alkyl aldehydes or carboxylic acids such as octyl or dodecyl aldehyde or carboxylic acid.

Finally, in order to increase its hydrophobicity and its ability to interact with fluorine-containing prosthetic materials (e.g., PTFE-containing materials), albumin or modified albumin may be halogenated, preferably fluorinated, by standard methods well known in the art. For example, albumin may be derivatized with polyfluoro dicarboxylic acid anhydrides (e.g., hexafluoro glutaric anhydride), polyfluoro aklyl ethers (e.g., perfluoroalkyl glycidyl ethers), or other halogen containing reagents.

Alternatively, a recombinant albumin may be produced by standard techniques of site-directed mutagenesis in which one or more amino acid residues are inserted, deleted or substituted to increase the viscosity of the albumin, to alter the hydrophobicity of the protein, to provide more side chains for derivatization, or to provide more free carboxyl or amine groups for the cross-linking reaction. As a general matter, under the conditions employed, albumin contains an adequate (and roughly equal) number of free carboxyl and amine groups for cross-linking. Therefore, it is anticipated that modifications of the albumin sequence will be most useful for increasing the viscosity of the protein by replacing small or hydrophilic residues (e.g., glycine, alanine) with larger and/or more hydrophobic and/or charged residues which can more participate in non-covalent intermolecular bonds through charge-charge or hydrophobic

interactions. Alternatively, however, one may produce two forms of modified albumin which differ substantially in their free carboxyl and amine contents. Such forms would tend to form intermolecular rather than intramolecular cross-links and would, therefore, have a greater degree of viscosity when mixed in roughly equal amounts.

II. Carbodiimide Cross-linkers

Carbodiimides are cross-linking reagents having the general formula:

$$R_1\text{-}N{=}C{=}N\text{-}R_2$$

where R_1 and R_2 may be essentially any chemical group, provided it does not contain a strong nucleophile. Carbodiimides are very reactive, and the presence of a nucleophilic group in either R_1 or R_2 would be destabilizing due to intermolecular (or intramolecular) reactions amongst carbodiimide molecules. In preferred embodiments, R_1 and R_2 are independently selected from the group consisting of any straight chain or branched, saturated or unsaturated, alkyl, alkenyl, aryl, aralkyl, or aralkenyl group, or variants thereof with halogen, tertiary amino, ester, keto, or other substituents. In addition, one or both of R_1 and R_2 may include an additional carbodiimide group, such that the cross-linker is a polycarbodiimide.

Preferably, carbodiimides are employed which are water soluble, and reactive with albumin under physiological conditions. However, a suspension of water insoluble carbodiimide, such as ethyl dimethylaminopropyl carbodiimide (EDC), may also be useful for albumin cross-linking if sufficiently dispersed in the cross-linking reaction. By appropriate choice of R groups, the solubility and reactivity of the carbodiimide may be varied. In addition, the choice of R groups will affect the immunogenicity and toxicity of the cross-linker, as well as its ability to interact with albumin molecules. In another embodiment, the R groups may be chosen to have additional cross-linking groups, such as photoactivatable cross-linking groups.

Preferably, the carbodiimides are provided as a solution or suspension. However, in some embodiments the carbodiimides may be provided in dry form, such as a powder. The dry carbodiimide is solubilized or suspended either before it is administered to the tissue, or by body fluids present at the site of administration.

One preferred cross-linking agent is ethyl dimethylaminopropyl carbodiimide hydrochloride (EDC·HCl). This cross-linking agent is water soluble, and effectively cross-links

albumin under conditions that are appropriate for bonding tissues and/or sealing surgical leaks *in vivo*.

Other examples of preferred carbodiimides include 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Aldrich catalog no. 42433-1); 1,3-di-*p*-tolylcarbodiimide (Aldrich catalog no. D21980-0); 1,3-diisopropylcarbodiimide (Aldrich catalog no. D12540-7); 1,3-dicyclohexylcarbodiimide (Aldrich catalog no. D8000-2); 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (Aldrich catalog no. C10640-2); polycarbodiimide (Aldrich catalog no. 45875-9); 1-tert-butyl-3-ethylcarbodiimide (Aldrich catalog no. 42639-3); 1,3-dicyclohexylcarbodiimide (Aldrich catalog no. 37911-5); 1,3-bis(trimethylsilyl)carbodiimide (Aldrich catalog no. 34433-8); 1,3-di-tert-butylcarbodiimide (Aldrich catalog no. 23556-3); 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (Aldrich catalog no. 16534-4); and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich catalog no. 16146-2), all available from the Aldrich Chemical Company, Milwaukee, WI.

In other preferred embodiments, the cross-linking agent is a polyethylene glycol (PEG) based water soluble carbodiimide of the general formula:

$$R_1O\text{-}PEG\text{-}R_2\text{-}N{=}C{=}N\text{-}R_3\text{-}PEG\text{-}OR_4$$

wherein R_1 through R_4 are independently selected as described above. For example, these can be –NCO (isocyanates) making it a bifunctional PEG and thus yeilding a poly(PEG carbodimide). R_2 and R_3 may also include hydrolytically or enzymatically cleavable groups so that a cross-linked product is biodegradable via cleavage of the crosslinks. A PEG based carbodiimide is synthesized by reacting alkoxy PEG isocyanates in the presence of an appropriate catalyst.

$$R_1O\text{-}PEG\text{-}R_2\text{-}NCO + R_4O\text{-}PEG\text{-}R_3\text{-}NCO \rightarrow R_1O\text{-}PEG\text{-}R_2\text{-}N{=}C{=}N\text{-}R_3\text{-}PEG\text{-}OR_4$$

Examples of useful catalysts are phospholenes, phospholanes, phospholene oxides or phospholene sulfides, phospholane oxides or phospholane sulfides.

In another preferred embodiment, the cross-linker (e.g., EDC·HCl) may be provided in an aqueous solution of an inert polymeric material. Preferably, the cross-linker solution and albumin solution have similar viscosities and volumes in order to promote efficient mixing and delivery. Examples of inert polymers include poly(vinyl alcohol), PEG, non-ionic surfactants, including PEG-based surfactants such as pluronic polymers, poly(saccharides) and other inert polymers.

III. Cross-linking reaction conditions

The carbodiimide cross-linkers of the invention can react spontaneously with albumin (and other proteins) to form cross-links between amino acid side chains of the proteins. Thus, initiators of the cross-linking reaction are not required. Importantly, the reaction can occur under conditions of pH and temperature which are compatible with *in vivo* applications.

The carbodiimide cross-linking reaction of the invention is very sensitive to pH, and is preferably conducted at a pH between 5 and 7, more preferably between pH 5 and 6. At pH values below 5, unmodified albumin may precipitate, and at pH values above 8, the speed of the cross-linking reaction is greatly reduced. Physiological pH is approximately 7.0-7.4 and, therefore, the reaction mixture may be allowed to occur at physiological pH, or the reaction mixture may be slightly acidified by the addition of acidic accessory molecules.

The reaction may be conducted at room temperature for *in vitro* uses, and may be conducted at body temperature for *in vivo* applications.

The molar ratio of the carbodiimide to albumin will significantly affect both the rate of the cross-linking reaction and the characteristics of the final cross-linked product. That is, the higher the ratio carbodiimide to albumin, the faster the reaction will occur and the more highly cross-linked the product will be.

The bioadhesives, surgical sealants and implantable devices of the invention are preferably prepared by mixing a carbodiimide with an albumin at a molar ratio of between 1 and 100 moles of carbodiimide per mole of albumin monomer. In a preferred embodiment, carbodiimide and albumin are mixed at a molar ratio of approximately 36:1. In a more preferred embodiment, the ratio is approximately 18:1. In some embodiments, sufficient cross-linking can be achieved with a carbodiimide:albumin ratio of approximately 4:1. However, a ratio of approximately 0.4:1 does not provide sufficient cross-linking for most applications of the invention. In an exemplary embodiment of the invention, consider a bioadhesive or surgical sealant produced using the carbodiimide . EDC·HCl has a molecular weight of approximately 191.7, whereas an albumin monomer has a molecular weight of, on average, about 69,293. Therefore, a bioadhesive prepared by mixing EDC·HCl with BSA at a molar ratio of approximately 36:1 corresponds to a mixture with approximately (36 moles x 191.7 grams/mole) 6901.2 g of EDC·HCl per (1 mole x 69,293 grams/mole) 69,293 g of BSA, or approximately 1:10 by weight EDC·HCl:albumin. Similar calculations may, of course, be used for other

carbodiimide reagents to determine the appropriate weight:weight ratios based on the desired molar ratios.

Thus, for example, EDC·HCl cross-linked albumin preparations may be produced with weight:weight ratios of EDC·HCl:albumin varying from about 1:1 to 1:100, preferably about 1:5 to 1:50, and most preferably about 1:10 to 1:20. At the ratio of 1:1, the reaction proceeds extremely rapidly and the product may cure before application has been completed. The resulting product is extremely tough and relatively non-plastic or inflexible. At a ratio of 1:20, the reaction is much slower and the product may be shaped and molded while the reaction proceeds. The resulting product is weaker, but is more plastic or flexible. A ratio of 1:100 is believed to be useful for some applications, but ratios as low as 1:1000 are expected to be inoperative due to insufficient cross-linking.

For applications involving, for example, the sealing of fluid and gas leaks in lung tissue, a weight:weight ratio of EDC·HCl:albumin of 1:5 is currently preferred. For applications involving larger films to seal wound openings, a lower EDC·HCl ratio, perhaps 1:10 to 1:20 may be appropriate, although this will produce a weaker and more gel-like sealant. A 1:20 ratio is expected to be too weak for cardiovascular applications, but a 1:1 ratio is expected to be too inelastic.

As noted above, in preferred embodiments, both the albumin and carbodiimide are provided in aqueous preparations. Alternatively, however, an aqueous preparation of albumin may be mixed with a suspension of an insoluble carbodiimide. Because the two components, albumin and carbodiimide, will begin to cross-link upon mixing, and because the reaction may be quite rapid for some formulations, it may be important to mix them immediately before application, or to mix them at the site where the bioadhesive or surgical seal is desired to be formed. In addition, because carbodiimides are not stable for extended periods in aqueous solution, they may be prepared by dissolving carbodiimide powder or lyophilized carbodiimide in an aqueous solution immediately prior to mixing with albumin. Alternatively, carbodiimides may be provided in an inert, water miscible, biocompatible organic solvent. Therefore, a binary delivery device, having separate compartments holding the albumin preparation and the carbodiimide cross-linker prior to dispensing and mixing, may be particularly useful. Thus, in one preferred method, a double-barreled syringe which simultaneously dispenses and mixes the components is used. Such a double-barreled syringe may be quite convenient for *in vivo*

applications where the bioadhesive or surgical sealant is applied to the site of tissue injury or incision. In one embodiment, a double-barreled syringe comprises a first barrel containing an aqueous solution of albumin, and a second barrel containing a carbodiimide powder separated from an aqueous solution by a breakable membrane. To employ such a syringe, the membrane is first broken, causing the carbodiimide to dissolve in the aqueous solution of the second barrel, and then the syringe is used as described above. In another embodiment, a double barrel comprises a first barrel containing an aqueous albumin solution at a pH at which the cross-linking reaction may occur (e.g., pH 5.0-6.0), and a second barrel containing a carbodiimide solution which has been adjusted to an alkaline pH to reduce the conversion of the carbodiimide to disubstituted urea compounds by water in the solution. In this embodiment, the pH and or buffer systems in the two barrels must be selected such that, upon mixing, the pH of the resultant solution is sufficiently acidic to permit the cross-linking reaction to proceed efficiently. In an alternative embodiment, a single barreled syringe contains an albumin solution separated from a carbodiimide powder by a breakable membrane. The cross-linking reaction is started by breaking the membrane, and the resulting mixture is applied as described above. For surgical uses, the two components and syringe may be provided in a sterile and disposable kit. Alternatively, the two components may be applied as a spray from a device with separate reservoirs for the two components. Finally, although it is not preferred, the two components may be applied sequentially. This method suffers from the disadvantage that the components will not be as thoroughly mixed, and only a thin coat of cross-linked albumin may form at their interface.

IV. Accessory Molecules

In some embodiments, accessory molecules are added to the reaction. Such accessory molecules may include viscosity-enhancing agents, solubility-enhancing agents, non-carbodiimide cross-linking agents, anti-inflammatory agents, hormones, growth factors, antibiotics, buffers, and the like.

In some preferred embodiments, viscosity-enhancing agents are added to the mixture and, therefore, the concentration of albumin which is employed may be decreased. However, the concentration of albumin is preferably at least 10%, and more preferably at least 20%. In preferred embodiments the viscosity-enhancing agent is itself cross-linked in the reaction. Viscosity-enhancing agents may include substituted or unsubstituted polysaccharides (e.g.,

glycosaminoglycans or heparin sulfates), fibrous proteins (e.g., collagen, elastin, fibrin, fibrinogen, thrombin, laminin), or other compounds which polymerize under physiological conditions or in the presence of the carbodiimides of the invention (e.g., polyacids and polyamines). Preferred viscosity-enhancing agents include glycosaminoglycans, dextran, hyaluronic acid, collagen, chondroitin sulfate, and elastin.

In some preferred embodiments, accessory molecules are added in order to alter the rate and/or degree of cross-linking. In general, a carboxylic acid may reduce the rate or degree of cross-linking by competing with a protein carboxylic group in the first step of the carbodiimide cross-linking reaction. Similarly, an amine may reduce the rate or degree of cross-linking by competing with a protein amine group in the second step of the carbodiimide cross-linking reaction. However, polycarboxylic acids, polyamines, poly(carboxy/amino)compounds (i.e., compounds having a multiplicity of carboxyl and amino groups), and mixtures thereof, may increase the rate of gel formation by reacting with carbodiimides to form cross-links with two or more protein molecules, thereby participating in the gel formation such polycarboxylic acids, polyamines, and/or poly(carboxyl/amino)compounds should have a relatively high density of carboxy and/or amino groups. Thus, such accessory molecules have molecular weights that are preferably less than 1,000, more preferably less than 500, and most preferably less than 250 Daltons per carboxy and/or amino group. For example, an accessory molecule having a molecular weight of 2,000 Daltons and having four carboxy and/or amino groups would have a molecular weight of 500 Daltons per carboxy or amino group. Polycarboxylic acids include citric acid and poly(acrylic acid). Polyamines include poly(lysine) and chitosan.

In alternative embodiments, other acids may be included to accelerate the EDC mediated protein cross-linking reaction by lowering the pH, and other bases may be used to slow the cross-linking reaction by raising the pH. The optimal pH for a carbodiimide cross-linking reaction is about 5.0 to 6.0. However, the reaction rate may also be increased at lower pH due to denaturation of the protein at the low pH.

In further embodiments, the hydrophobicity of the albumin solution is increased by solubilizing the albumin in a solution that is more hydrophobic than water. In a preferred embodiment, the albumin is solubilized in a solution comprising a secondary or tertiary alcohol. Preferably, albumin is provided in a solution of isopropyl alcohol (IPA) or isobutyl alcohol (IBA). Most preferably, a 30% solution of BSA is prepared with 20% IPA or 8% IBA.

Finally, because they react with carbodiimides, buffers such as phosphate buffers and Tris buffers are not preferred. Currently preferred buffers are inorganic acids such as hydrochloric acid.

V. Utilities

The cross-linked albumin preparations of the invention have a large number of applications. For example, they may be employed as surgical sealants, bioadhesives, hemostats, coating materials and drug delivery media. When formed *ex vivo* into films and then implanted, they may also serve to prevent surgical adhesions by forming a resorbable barrier between healing tissues. For use as surgical sealants, the cross-linked albumins are expected to be particularly useful in sealing air and blood leaks in the lungs, blood leaks in the liver, spleen and kidneys, cerebrospinal fluid leaks in dura, and blood leaks in the heart and vascular system.

For cardiovascular applications, the carbodiimide cross-linked albumins must be designed to have sufficient flexibility for pulsatile stretching, but must also have sufficient strength to withstand cardiovascular pressures. In preferred embodiments, the reaction mixture rapidly forms a seal when it is applied to a vascular leak. Preferably, cross-linking occurs before the reaction mixture can slide off or wash away from the site of vascular application, for example a blood vessel. In addition, cross-linking preferably occurs before the reaction mixture is diluted or washed away by blood or other body fluids. In a preferred embodiment, a cross-linked gel forms within 5 minutes from the time at which the components are mixed and applied to the vascular site. In a most preferred embodiment, the cross-linked gel forms within 30-45 seconds.

Rapid gel formation is also important to prevent excessive blood loss. This is especially important when a patient, such as a trauma patient in an emergency situation, has multiple sites of vascular injury. In one embodiment, the reaction mixture may be applied to stop bleeding when the precise source of blood loss has not been identified. In this embodiment, the gel formation time should be slow enough for the reaction mixture to be applied over a relatively large area of bleeding, but fast enough to seal the leak or leaks before the reaction mixture is diluted or washed away.

In a pulmonary application, the gel reaction mixture is used to seal an air or blood leak in a lung. In a preferred embodiment, the reaction mixture is designed to take longer to form a gel than a reaction mixture that is used for a vascular application. Preferably, the reaction mixture

may be spread across the surface of the lung before it cross-links to form a gel. In addition, the reaction mixture may contain additives such as lipids or surfactants that help spread the mixture over the lung surface. In a pulmonary application, the mixture is less likely to slide off or wash away from the application site, because the lung provides a relatively large and flat surface area when compared to the surface area of a blood vessel.

In a preferred embodiment, a primer solution is applied to the tissue before the gel mixture. Preferably, the primer solution is a dilute solution of BSA. More preferably, the primer solution is a dilute solution of the cross-linking reaction mixture without the cross-linker. In the embodiment, the primer mixture is hydrophobic. In one embodiment, a hydrophobic derivation of albumin is used. In another embodiment, albumin is mixed with a fatty acid. For example, albumin may be complexed with palmityl anhydride. In a preferred embodiment the molar ratio of fatty acid to albumin is greater than 1:1, and preferably 3:1. Alternatively, albumin may be purchased in association with a fatty acid. For example, BSA may be purchased in association with octanoic acid (Sigma catalog number A3174, 10-15mg octanoic acid per g of protein). In a preferred embodiment, the primer solution is applied to the tissue to replace body fluids and provide a good environment for the cross-linking reaction. A brush is a useful applicator to spread the primer at the tissue location where the cross-linking reaction mixture will be administered.

In some embodiments, the albumin solution being cross-linked comprises additional reagents to promote interaction with the tissues at the site of application. In a preferred embodiment, an albumin solution comprises a surfactant and/or a lipid when it is used in a pulmonary context. Preferably, the surfactant and lipid component is similar to the natural surfactant and lipid composition of the lung. Alternatively, synthetic surfactants and lipids may be used.

The cross-linked albumin compositions of the invention may also be used to produce implantable drug delivery devices. In particular, because the cross-linked albumin compositions will be slowly eroded and resorbed by the body, the cross-linked albumin compositions may be used to produce bioerodable implants which contain drugs interspersed throughout the cross-linked matrix of albumin. The degree of cross-linking will determine both the ability of drugs to diffuse into and out of the matrix, and the rate at which the device erodes. In addition, the degree of cross-linking will determine the rigidity or flexibility of the device. By controlling the degree

of cross-linking, therefore, devices may be made which deliver drugs at different rates, and which have different degrees of flexibility. The devices are produced by introducing an albumin preparation and carbodiimide of the invention, optionally with accessory molecules such as pharmaceuticals, into a mold under conditions which promote cross-linking of the albumin to form the implantable device.

EXAMPLES

Example 1. The Effect of pH on the Rate of BSA Cross-linking with EDC·HCl

A 37.5% solution of BSA was prepared for the following cross-linking experiments by dissolving BSA (6 g) in distilled water (10 ml), and stirring for several hours to get a clear solution. A 1 ml aliquot of this solution was cross-linked with 0.25 ml of an 8% aqueous EDC·HCl solution at different pH values.

Experiment 1: pH 7.0

A 1 ml solution of 37.5% BSA (pH = 7.09) was mixed with 0.25 ml of 8% EDC·HCl (pH = 7.0) as follows. The protein solution was stirred with a small magnetic stir bar, using a magnetic stirrer at a constant setting. The solution was viscous and the bar stirred slowly. When the EDC·HCl solution was added, stirring became faster, presumably due to the addition of more water and the consequent dilution of the BSA. After 10 minutes, the stirring became progressively sluggish, presumably due to the EDC·HCl promoted cross-linking of the BSA. By 15 minutes, stirring had stopped completely. The cross-linked gel was soft but rubbery, and showed considerable cohesion.

Experiment 2: pH 6.02

A solution of BSA at pH 6.02 was prepared by adding drops of 0.5 N HCl to 1 ml of 37.5 % BSA dissolved in water. As described above, 0.25 ml of 8% EDC·HCl was added while the solution was stirring. Stirring stopped 1 minute after complete addition of EDC·HCl, and a very cohesive, rubbery, non-tacky gel was produced.

Experiment 3: pH 5.5

A solution of BSA at pH 5.5 was prepared by adding drops of 0.5 N HCl to 1 ml of 37.5 % BSA dissolved in water. As described above, 0.25 ml of 8% EDC·HCl was added while the solution was stirring. Stirring stopped after 31 seconds, and again a very cohesive gel was produced.

Experiment 4:

A similar experiment was performed with a 1 ml solution of 37.5% BSA at pH 5.3. In this experiment, a strong gel was formed within 26 seconds.

The total volume of the gel was 1.25 ml. The gel contained 0.375 g of BSA, 0.020 g of EDC·HCl, and 0.855 g of water. Solids therefore represented 31.6 % of the gel weight, with EDC·HCl representing only 1.6 % of the gel weight. The cross-linked protein contained approximately 5.3 % EDC·HCl by weight.

These experiments suggest that the rate of BSA cross-linking by EDC·HCl is higher at lower pH, within the pH range that was tested. The relative cross-linking rates at different pHs are ranked as follows: 5.3>5.5>6.01>>7.

Example 2. Cross-linking of a Commercial 30% BSA Solution with EDC·HCl

A 30% solution of BSA was purchased from Sigma (catalog number A8327). The pH of 1.5 ml of this BSA solution was adjusted to between 5.38 and 5.40 with 3 drops of 0.5 N HCl. A 1 ml aliquot of the pH adjusted BSA was stirred with a magnetic bar using a stirrer at a constant setting. A 0.25 ml volume of an 8% EDC·HCl solution (80 mg EDC·HCl dissolved in 1 ml water) was added. Stirring became sluggish after 1 minute, and stopped after 1½ minutes. The resulting gel was less rigid than the gel obtained using a 37.5% BSA solution.

Example 3. A Two Syringe Mixing System for Delivering BSA/EDC·HCl Bioadhesive

A two syringe mixing system delivering a 9.3:1 ratio of BSA:EDC·HCl was tested. A 3 ml volume of BSA at pH 5.5 taken up in a first syringe (a 10 cc syringe). A 0.5 ml volume of 18.12% EDC·HCl (90 mg EDC·HCl dissolved in 0.500 ml of distilled water) was taken up in a second syringe (a 1 cc syringe). The syringes were connected to a modified Micromedix (Eagan, MN) applicator mixing nozzle. The ends of the syringe plungers were attached to each other with a plastic tab, and a mixture of BSA and EDC·HCl was extruded into a PE weigh boat. The mixture came out as a non-viscous solution which rapidly gelled into a non-flowing gel in about 40 seconds. This gel was still soft to the touch. After about 1½ minutes, the mixture was completely cured as a rubbery, highly cohesive cross-linked gel.

Example 4. The Effect of BSA and EDC·HCl Concentration on the Rate of BSA Cross-linking with EDC·HCl

A 1 ml aliquot of a 30% solution of BSA solution was cross-linked with 0.125 ml of a 16.67% aqueous EDC·HCl solution at pH 7. The solution gelled within 11-12 minutes.

A similar experiment, when conducted with a 35% solution of BSA, resulted in a slightly slower gelling time of 13-15 minutes.

In another experiment, a 1 ml aliquot of a 30% solution of BSA was cross-linked with 0.125 ml of an aqueous 23.07% EDC·HCl solution at pH 7. The solution gelled within 7-8 minutes. However, when the experiment was performed at a 50% concentration of BSA at pH 7, the gelling was faster and a gel was obtained in 4-6 minutes.

A 1 ml aliquot of a 30% solution of BSA was cross-linked with 0.125 ml of an aqueous 33.34% EDC·HCl solution at pH 7. The solution gelled within 5-6 minutes, thus indicating that a higher concentration of the crosslinker results in a shorter gel time.

Example 5. The Effect of Additives on the Rate of Gel Formation

A 1 ml volume of a 35% solution of BSA and glutaric anhydride-derivatized BSA (a polyacid), in a ratio of 3.6:1 at pH 5.8, was mixed with 0.125 ml of an aqueous 16.6% solution of EDC·HCl, resulting in a self standing rubbery gel after 15-25 seconds. In a similar reaction, a 35% solution of BSA (without any glutaric anhydride derivatized BSA) gelled after 55-65 seconds.

In another experiment, a 1 ml volume of a 35% solution of BSA (pH 5.5) was mixed with 0.1 ml of deionized (DI) water and 0.15 g of citric acid solid (the final BSA concentration was 28-30%). When mixed with 0.125 ml of an aqueous 16.67% solution of EDC·HCl, a rigid gel was obtained within 4-8 seconds. In a similar reaction without the citric acid, the gel time was 30-40 seconds.

As discussed above, polyacids such as dicarboxylic acids, citric acid, polyacrylic acid, glutaric anhydride derivatized BSA, and other polyacids, are able to accelerate the gelation either by changing the local pH, by reacting with EDC·HCl at a faster rate than unmodified BSA, or by denaturing the BSA and thereby increasing the rate of BSA cross-linking. However, a monocarboxylic acid in high proportions may result in a longer gel time by interfering with the protein cross-linking reaction.

In a further experiment, 1 ml of 35% BSA was mixed with 0.1 ml of an aqueous 20% solution of EDC·HCl, resulting in a rigid gel in 45-50 seconds. In a similar experiment, 0.6% of ethylene diamine dihydrochloride was added along with the cross-linker, and the gelation was slowed to 70-85 seconds. Thus, a diamine can reduce the rate of gelation. However, a polyamine (e.g., polyethylene amine, chitosan, poly(l-lysine)) may be expected to accelerate the reaction. Other additives, such as N-hydroxy succinimide (NHS), a water soluble analog (sulfo NHS, the sulfonic acid salt of NHS), or hydroxy benzotriazole (HOBT) were shown to reduce the rate of gelation. These effects were pH sensitive.

In one experiment a 4% solution of Dextran (molecular weight: 3-7 M) was made in a 35% BSA solution (pH 5.5). When mixed with a 16.67% aqueous solution of EDC·HCl at a ratio of 8:1 this very thick solution yielded a rigid gel within 25 seconds.

In another experiment, 2g of BSA was dissolved in 5ml of a slurry of 25% hydroxyapatite. The pale yellow, milky solution was mixed with a 16.67% aqueous solution of EDC·HCl and poured quickly into a cylindrical mold. The solution rapidly gelled, yielding a strong flexible material that could be used as a matrix for plastic surgery, tissue engineering applications, or drug delivery systems for example.

Example 6. Shelf-life of EDC·HCl

In experiments designed to assess the shelf-life of EDC·HCl both at room temperature (RT) and refrigerated, as well as both as a solid and an aqueous solution, a 16.67% aqueous solution of EDC·HCl was used to achieve gelation of a 30% (pH5.5) BSA solution. The gel time was considered as a direct measure of the activity of the crosslinker. Accordingly, the crosslinker can be provided as a powder that can be stored at room temperature for over 6 months. Alternatively, an aqueous solution of cross-linker should be refrigerated, and is fully active for less than one month.

Solid crosslinker at RT(24 deg C) in air-tight containers stored in a desicator:

Shelf-Time	Gel Time
0	25-30 seconds
1 week	25-30 seconds
1 month	22-25 seconds
3 months	25-30 seconds
6 months	27-35 seconds

Aqueous solution of crosslinker (16.67%) in refrigerator :

Shelf-Time	Gel Time
0	25-30 seconds
1 week	25-30 seconds
1 month	36-40 seconds
3 months	1-2 minutes
6 months	> 24 hours

Aqueous solution of crosslinker (16.67%) at RT (24 deg C):

Shelf-Time	Gel Time
0	18-23 seconds
5 min	18-23 seconds
10 min	18-23 seconds
1 hour	18-23 seconds
2 hours	20-25 seconds
18 hours	20-25 seconds
42 hours	25-35 seconds
1 week	60-75 seconds

Example 7. Adhering an End-to-Side Arterial Anastomosis of ePTFE Graft to Artery

In a first experiment, a sealant mixture of BSA and EDC·HCl was delivered *in vitro* onto an end-to-side anastomosis of an ePTFE (expanded polytetrafluoroethylene) graft onto a porcine aorta. The mixture was delivered through a static mixing nozzle, and contained a 9.3:1 ratio of

35% BSA (pH 5.55) : 40% EDC·HCl. The glue mixture was slowly extruded onto all sides of the anastomosis. The mixture cross-linked fairly rapidly. Indeed, the gel could be neatly cut with scissors ~ 30 seconds after its application. The artery was pressurized by introducing saline via a large syringe, and the glue treated graft provided a good seal.

In another experiment a 40% solution was prepared by using a 25/10 ratio of BSA and glutaric anhydride derivatized BSA. This solution (pH 6) was used with a 16.67% aqueous solution of EDC·HCl at a ratio of 8:1 (vol/vol) on a porcine lung (ex vivo) to seal a planar wedge resection. The solution on curing adhered to the lung tissue and withstood a static air pressure in excess of 60 mm of Hg (average lung pressure during surgery is in the range of 20-25 mm of Hg).

The 9.3:1 ratio of BSA solution : EDC·HCl solution was produced as follows. A 5 ml volume of 35% BSA (pH 5.5) was taken up in a 10 cc syringe, and a 1 ml volume of 40% EDC·HCl (400 mg EDC·HCl dissolved in 1 ml water) was taken up in a 1 cc syringe. The two syringes were connected to a static mixing nozzle which delivered the BSA and EDC·HCl solutions in a 9.3:1 ratio. Assuming total delivery of the BSA solution, the resulting gel contains 5.0 ml i.e. 5 x 35% = 1.75 g BSA. The gel also contains 5/9.3 = 0.538 ml = 0.538 x 40% = 0.215g EDC·HCl (the EDC·HCl syringe is not emptied, only 0.538 ml of the EDC·HCl solution is delivered with the 5 ml volume of BSA solution). The total weight of the glue is 5.538 g, therefore the % EDC·HCl in the glue by weight is 0.215 x 100 / 5.538= 3.88%. The % EDC·HCl in albumin by weight is 0.215 x 100 / 1.75 = 12.3%.

In a second experiment, the same composition was used on a 2 x 20 mm ePTFE patch sewn into a porcine carotid artery *in vivo*. The albumin sealant adhered to the vessel and sealed the leaking suture line.

Example 8. Effect of Derivatization of BSA

BSA was derivatized to increase its hydrophobicity with reactive molecules with hydrophobic tails.

In one experiment, 10 g of BSA was dissolved in 200 ml of 0.05N phosphate buffer and pH adjusted to 8.5. 6.89 ml of hexanoic acid anhydride was added in an acetone solution (the acetone solution is saturated with hexanoic acid anhydride) at once. There was no obvious change in the pH of the solution. The reaction was allowed to stir for 2 days at low temperature.

The mixture was diafiltered, pH adjusted to 6.0, and dried. The dry derivatized BSA exhibited a more hydrophobic nature as evidenced from contact angle studies.

In one experiment, 10 g of BSA was dissolved in 90 ml of de-ionized water and 100 ml of 0.05N phosphate buffer and pH adjusted to 8.5. 13g of pyromellitic dianhydride was added in an acetone solution dropwise. The pH was maintained at 8-9 using dilute NaOH. The reaction was allowed to stir overnight at low temperature (approximately 4 °C). The mixture was diafiltered, pH adjusted to 6.0, and dried. The dry derivatized BSA exhibited a more hydrophobic nature as evidenced from contact angle studies. The solution was also highly viscous as compared to BSA solutions of similar concentrations.

In one experiment, 10 g of BSA was dissolved in 67 ml of 0.05N phosphate buffer. The pH of the resulting solution was adjusted to 8.5, and 6 g of tetra fluoro phthalic anhydride was added as a solution in acetone. The pH was maintained at 8-9 using dilute NaOH. After several hours of stirring, the reaction mixture was diafiltered, pH adjusted to 6.0, and dried. The dry derivatized BSA exhibited a more hydrophobic nature as evidenced from contact angle studies.

In one experiment, 20 g of BSA was dissolved in 500ml of a 65/35 mixture of de-ionized water and methanol. The pH of the pale yellow solution was adjusted to 9.0, and 8 ml of (2, 2, 3, 3, 4, 4, 5, 5, 6, 6, 7, 7, 8, 8, 9, 9, 9-heptadecafluorononyl)-oxirane was added all at once in a 50% acetone solution. The reaction was allowed to stir for 2 days, maintaining the pH at 9. The slightly turbid solution was centrifuged, dialyzed, pH adjusted to 6.0, and allowed to dry. The dry modified BSA solution exhibited higher viscosity and an improved wettability towards ePTFE graft, and on cross-linking with the appropriate amount of EDC·HCl, adhered very well to the graft and natural tissue.

Example 9. Dry Powder Formulations

In one experiment, Gelatin (300 Bloom, Sigma) was thoroughly mixed with EDC·HCl in a ratio of 10:1. This powder was applied on a canine lung in a planar wedge resection model. The material absorbed the neighboring fluids, rapidly gelled into a rubbery mass, and proceeded to stop the leak.

Example 10. Lung Applications

A 40% (pH 6) BSA solution was mixed with Tyloxapol and dipalmitoyl, phosphatidyl choline (DPPC) such that they were 1mg/ml and 14 mg/ml, respectively. The dispersion was

mixed (10: 1) with an aqueous solution of EDC HCl (20%) and applied to a porcine lung in a planar wedge resection model. The site was previously primed with a 30% solution of BSA (pH 5.5). The material was allowed to attain maximum strength (about 4-5 minutes), and then tested. The material withstood a dynamic pressure of about 100mm of Hg before lung tissue rupture occurred.

In another experiment, Gelatin (300 Bloom) was mixed with DPPC and tyloxapol in a similar ratio. The material was a gel at room temperature. The material was warmed to about 45 ° C and mixed appropriately with an aqueous EDC·HCl solution. On applying to the lung tissue, the material gelled rapidly and adhered satisfactorily to the wound site.

CLAIMS

What is claimed is:

1. A method for producing a cross-linked albumin composition for use in a bioadhesive, surgical sealant or implantable device, comprising the steps of:
(a) providing an albumin preparation;
(b) providing a carbodiimide preparation; and
(c) mixing said albumin preparation and said carbodiimide preparation under conditions which permit cross-linking of said albumin.

2. A method as in claim 1, wherein said albumin preparation comprises a protein selected from the group consisting of a naturally occurring albumin protein, a recombinant albumin protein, a major fragment of an albumin protein, and a chemically modified albumin.

3. A method as in claim 2, wherein said albumin is selected from the group consisting of a mammalian albumin protein and a major fragment of a mammalian albumin protein.

4. A method as in claim 2, wherein said albumin preparation comprises a protein comprising an amino sequence of at least 100 amino acid residues having at least 60% homology to an amino acid sequence of human albumin.

5. A method as in claim 2, wherein said albumin comprises an amino-acid sequence which has been recombinantly modified relative to a naturally occurring albumin sequence to enhance one or more physical properties selected from the group consisting of solubility, reactivity with a carbodiimide cross-linker, stability, viscosity in an aqueous solution, and immunocompatibility.

6. A method as in claim 2, wherein said albumin comprises an albumin which has been covalently bound to a molecule selected from the group consisting of polysaccharides (e.g., glycosaminoglycans, dextrans, hyaluronic acid, chondroitin sulfate, heparan sulfates), polyethers (e.g., polyethylene glycol, polypropylene glycol, polybutylene glycol), polyesters (e.g., polylactic acid, polyglycolic acid, polysalicylic acid), and aliphatic, alicyclic, aromatic, perfluorinated or non-perfluorinated, acylating or sulfonating agents.

7. A method as in claim 2, wherein said albumin preparation is chlorinated, fluorinated, brominated or iodinated.

8. A method as in any one of claims 1-7 wherein said albumin preparation is in an aqueous solution at a concentration of about 10-50 % by weight.

9. A method as in any one of claims 1-7 wherein said albumin preparation is in an aqueous solution at a concentration of about 20-40% by weight.

10. A method as in any one of claims 1-7 wherein said albumin preparation is in an aqueous solution at a concentration of about 35-40% by weight.

11. A method as in any one of claims 1-10, wherein said carbodiimide preparation comprises a carbodiimide having a general structure R_1-N=C=N-R_2 wherein R_1 and R_2 are independently selected from the group consisting of straight chain or branched, saturated or unsaturated, alkyl, alkenyl, aryl, aralkyl, or aralkenyl groups, or variants thereof with halogen, tertiary amino, ester, keto substituents or other degradable groups.

12. A method as in claim 11 wherein said carbodiimide is selected from the group consisting of ethyl dimethylaminopropyl carbodiimide (EDC·HCl); 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; 1,3-di-*p*-tolylcarbodiimide; 1,3-diisopropylcarbodiimide; 1,3-dicyclohexylcarbodiimide; 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate; polycarbodiimide; 1-tert-butyl-3-ethylcarbodiimide; 1,3-dicyclohexylcarbodiimide; 1,3-bis(trimethylsilyl)carbodiimide; 1,3-di-tert-butylcarbodiimide; 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide; and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

13. A method as in any one of claims 1-10, wherein said albumin and carbodiimide are mixed at a carbodiimide:albumin weight ratio between approximately 1:1 and 1:100.

14. A method as in any one of claims 1-10, wherein said albumin and carbodiimide are mixed at a carbodiimide:albumin weight ratio between approximately 1:5 and 1:50.

15. A method as in any one of claims 1-10, wherein said albumin and carbodiimide are mixed at a carbodiimide:albumin weight ratio between approximately 1:10 and 1:20.

16. A method as in any one of claims 1-10, wherein said albumin and carbodiimide are mixed at a carbodiimide:albumin molar ratio between approximately 100:1 and 1:1.

17. A method as in any one of claims 1-10, wherein said albumin and carbodiimide are mixed at a carbodiimide:albumin molar ratio between approximately 36:1 and 4:1.

18. A method as in any one of claims 1-10, wherein said albumin and carbodiimide are mixed at a carbodiimide:albumin molar ratio of approximately 18:1.

19. A method as in any one of claims 1-10, wherein said mixing step is performed at a tissue locus.

20. A method as in any one of claims 1-10, wherein an accessory molecule is provided and mixed with said albumin and carbodiimide preparations.

21. A method as in claim 20, wherein said accessory molecule is selected from the group consisting of viscosity-enhancing agents, cross-linkers, buffers, hormones, growth factors, antibiotics, surfactants, lipids, fatty acids, and anti-inflammatory agents.

22. A method as in any one of claims 1-10, wherein said albumin preparation comprises a secondary or a tertiary alcohol.

23. A method as in claim 21, wherein the albumin preparation comprises IPA or IBA.

24. A method for adhering a first biological tissue to a second tissue and/or prosthetic device, the method comprising

contacting said first tissue and said second tissue or prosthetic device with a mixture of an albumin preparation and a carbodiimide preparation under conditions that promote cross-linking of said albumin preparation to said first tissue and said second tissue or prosthetic device.

25. A method for sealing incisions, perforations, and/or fluid or gaseous leaks in biological tissues during a surgical procedure, comprising

contacting said tissue with an effective amount of an albumin preparation and a carbodiimide preparation under conditions that promote cross-linking of said albumin preparation to said tissue and thereby seal said incision, perforation, or fluid or gaseous leak.

26. A method as in claim 25, wherein said surgical procedure is selected from the group consisting of cardiovascular, pulmonary, renal, and hepatic surgeries.

27. A method for forming an implantable device comprising

(a) providing an albumin preparation;

(b) providing a carbodiimide preparation;

(c) providing a mold; and

(d) mixing said albumin preparation and said carbodiimide preparation under conditions which permit cross-linking of said albumin in said mold.

28. A bioadhesive, surgical sealant or implantable device produced according to any of the foregoing methods.

29. A kit for producing a bioadhesive, surgical sealant or implantable device comprising, in separate containers,
a) an albumin preparation; and
b) a carbodiimide preparation.

30. The kit of claim 29, further comprising an accessory molecule.

31. The kit of claim 30, wherein said accessory molecule is selected from the group consisting of viscosity-enhancing agents, cross-linkers, buffers, hormones, growth factors, antibiotics, and anti-inflammatory agents.

32. The kit of claim 29, wherein said albumin preparation and said carbodiimide preparation are provided in a binary delivery device that delivers said preparations in a ratio which is either predetermined or regulatable.

33. A method as in any one of claims 24-26, wherein any one of said tissues or prosthetic device is first contacted with a primer solution.

34. A method as in claim 33, wherein said primer solution is a saline solution.

35. A method as in claim 33, wherein said primer solution is an albumin solution.

36. A method as in claim 35, wherein said albumin solution is identical to said albumin preparation.

37. A method as in claim 36, wherein said albumin solution is a dilute albumin preparation.

38. A method as in any one of claims 1-10, 24-26, or 33-37, wherein said albumin and carbodiimide preparations are provided at a pH between 5.0 and 8.0.

39. A method as in claim 38, wherein said pH is between 5.5 and 7.5.

40. A method as in claim 39, wherein said pH is between 6.0 and 7.0.

41. A method as in claim 1, wherein said cross-linking reaction results in gel formation in under 10 minutes.

42. A method as in claim 41, wherein said gel formation occurs in under 5 minutes.

43. A method as in claim 41, wherein said gel formation occurs in under 1 minute.

44. A method as in claim 41, wherein said gel formation occurs in 30 seconds.

45. A method as in claim 12, wherein said carbodiimide preparation comprises an aqueous solution of between 10% and 20% EDC·HCl.

46. A method as in claim 45, wherein said carbodiimide preparation comprises an aqueous solution of 15% EDC·HCl.

47. An albumin preparation for cross-linking with EDC·HCl, comprising albumin and derivatized albumin, wherein the ratio of albumin to derivatized albumin is suitable for a rate of cross-linking that is adapted to a specific tissue application.

48. The albumin preparation of claim 47, wherein said specific tissue application is a cardiovascular application.

INTERNATIONAL SEARCH REPORT

Int' .ional Application No
PCT/US 99/14232

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAIDI BENSLIMANE, ROBERT GUIDOIN, PAUL-EMILE ROY, JUAN FRIEDE, JACQUES HÉBERT: "Degradability of crosslinked albumin as an arterial polyester prosthesis coating in in vitro and in vivo rat studies." BIOMATERIALS, vol. 7, 1986, pages 268-272, XP002117760 page 268, left-hand column, paragraph 1 page 268, right-hand column, paragraph 3 -page 269, left-hand column, paragraph 2 --- -/--	1-3,6,8, 9,11,12, 19-22, 24, 27-31, 38-40, 45,46

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 1999	18/10/1999

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 632 776 A (KURUMATANI HAJIMU ET AL) 27 May 1997 (1997-05-27) column 4, line 5 - line 50 column 5, line 33 - line 47 column 6, line 13 - line 30 ---	1-3,6, 8-11, 19-22, 24-31
X	EP 0 466 966 A (SEIKAGAKU KOGYO CO LTD) 22 January 1992 (1992-01-22) page 2, line 34 - line 49 page 2, line 56 -page 3, line 22 example 1 ---	1-3,6, 8-21, 24-32, 38,39, 45-47
X	US 4 046 871 A (RECKEL RUDOLPH P) 6 September 1977 (1977-09-06) column 2, line 30 - line 45 column 3, line 22 - line 51 column 6, line 45 -column 7, line 53 ---	1-3, 6-21,24, 29-31, 33-40, 45-47
X	US 4 526 714 A (FEIJEN JAN ET AL) 2 July 1985 (1985-07-02) column 1, line 7 - line 51 column 1, line 64 -column 2, line 15 example 1 ---	1-4,6,7, 11,12, 19-21, 24-31, 38,39, 45-47
X	EP 0 503 741 A (BALDACCI LAB SPA) 16 September 1992 (1992-09-16) page 2, line 16 - line 28 claims 1,3,5,6 ---	1-3,6, 11,12, 19-21
A	US 5 376 692 A (PARK KINAM ET AL) 27 December 1994 (1994-12-27) column 1, line 29 - line 38 column 6, line 31 - line 58 column 3, line 3 - line 12 -----	1-5, 8-10, 19-31, 38-40, 45-48

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INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 99/14232

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: –
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid. specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 1.1

Although claims 24,25,26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by surgery

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 24,25,26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by surgery

INTERNATIONAL SEARCH REPORT

information on patent family members

Int. ional Application No
PCT/US 99/14232

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5632776 A	27-05-1997	CA 2074362 A	23-05-1992
		EP 0512122 A	11-11-1992
		WO 9209312 A	11-06-1992
EP 0466966 A	22-01-1992	JP 2191601 A	27-07-1990
		AU 625386 B	09-07-1992
		AU 5915790 A	23-01-1992
US 4046871 A	06-09-1977	AT 357270 B	25-06-1980
		AT 252976 A	15-11-1979
		DE 2614865 A	21-10-1976
		DK 146576 A	09-10-1976
		FI 760934 A,B,	09-10-1976
		FR 2307269 A	05-11-1976
		GB 1537871 A	04-01-1979
		JP 51123817 A	28-10-1976
		SE 7602536 A	09-10-1976
US 4526714 A	02-07-1985	US 4678671 A	07-07-1987
EP 0503741 A	16-09-1992	IT 1245217 B	13-09-1994
US 5376692 A	27-12-1994	NONE	